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14. ABSTRACT The induction of human breast cancer involves the complex interplay of hormones and growth factor receptors. The major focus of our DOD sponsored research program is to investigate the interaction of the ERα and erbB-2 receptor in the induction of breast cancer. In addition, we studied the involvement of ErbB-2 coupled signaling pathways such as c-Src in ERα induced mammary tumorigenesis. To accomplish this, we derived and characterized transgenic mice that expressed high levels of ERα in the mammary epithelium. To explore the significance of ErbB-2 and c-Src in mammary tumor progression, we generated cohorts of bigenic mice expressing MMTV-ERα along with ErbB-2, c-Src or Akt-1. The results of these analyses did not show any influence of the expression of an activated form of ERα on ErbB-2 induced mammary tumor formation. No evidence of c-Src or Akt-1 acting synergistically with ERα to accelerate mammary tumor progression has been observed either.					
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Introduction

The major goal of our DOD sponsored research program has been directed towards understanding the importance of the cross-talk between the ErbB-2 receptor tyrosine kinase and ER α receptors during mammary tumorigenesis. The rationale behind these experiments stems from the observation that in addition to a well documented role for ER α as a transcriptional activator, ER α has also been shown to mediate non genomic effects that are too rapid to involve transcription regulation and are thought to occur through the formation of physical complexes with the c-Src tyrosine kinase [1]. Since c-erbB-2 is also known to activate c-Src tyrosine kinase [2] [3], it is conceivable that c-Src may play a critical role in mediating the cross-talk between ER α and ErbB-2. To assess the role of ER α in ErbB-2 mediated tumorigenesis, we proposed to investigate whether the mammary epithelial specific expression of a constitutively active ER α could modulate mammary tumor progression in the ErbB-2 transgenic mouse strains. To do so, we derived transgenic mice that express a constitutively activated version of ER α under the transcriptional control of the MMTV promoter. We then generated cohorts of bigenic mice by interbreeding the MMTV-activated ER α mice with separate strains of transgenic mice expressing ErbB-2 under MMTV (NeuNDL2-5) [4] or its endogenous promoter (NeuNT) [5]. In addition, we also assessed whether ErbB-2 coupled signaling molecules such as c-Src and Akt could act synergistically with ER α to accelerate mammary progression. Therefore we generated a cohort of bigenic mice by interbreeding the MMTV-activated ER α mice with transgenic mice expressing an activated form of c-Src or Akt-1 under the control of the MMTV promoter.

Body

Mammary specific expression of ER α is not associated with mammary tumor induction.

As previously reported, we generated three independent transgenic mouse strains expressing high levels of ER α . As previously described, high expression of ER α has been shown by western-blotting, immunohistochemistry and real-time RT-PCR.

Despite the high levels of ER α expression in the mammary epithelium, females carrying the MMTV-activated ER α did not develop mammary tumors even at 20 months of age. These data suggest that mammary epithelial expression of ER α on its own is not sufficient to induce a mammary tumor phenotype.

Generation of bigenic animal co-expressing Neu, c-Src or Akt-1 with activated ER α in the mammary epithelium.

Given the observation that ectopic expression of ER α was not able to induce mammary tumors, we next asked if ER α could cooperate with other transgenic strains to accelerate mammary tumor induction. Table # 1 summarizes the various crosses that were examined. To assess whether co-expression of activated ER α and Neu could impact on tumor induction, the MMTV-ER α mice were interbred either with separate strains of mice carrying MMTV-activated erbB-2 (NeuNDL2-5) or activated erbB-2 under the control of its endogenous promoter (NeuNT). In addition to these crosses we have also generated transgenic crosses in which key downstream

signaling pathways such as c-Src and Akt-1 are co-expressed in the mammary epithelium with activated ER α . The number and range of ages of the transgenic mice generated are summarized in Table 1.

Table 1 : Summary of generated transgenic mice

Genotype	Number of females	Range of ages
aER α /NeuNDL2-5* ¹	18	21 to 31 weeks
aER α /NeuNT* ²	10	50 to 72 weeks
aER α /Src2* ³	13	36 to 62 weeks
aER α /Akt7* ⁴	10	28 to 52 weeks

*¹ Activated form of ErbB-2 under the control of the MMTV promoter

*² Activated form of ErbB-2 under the control of its endogenous promoter

*³ Activated form of c-Src under the control of the MMTV promoter

*⁴ Activated form of Akt-1 under the control of the MMTV promoter

All the mice have been monitored for tumor formation by a biweekly physical palpation. No tumor arose in any of the aER α /Src2, aER α /Akt7 or aER α /NeuNT mice during the year observation period. Given that MMTV/Akt mice fail to develop mammary tumors [6] and that MMTV/activated Src and activated Neu under the transcriptional control of its endogenous promoter fail to develop mammary tumors until well after 15 months [7] [8], the lack of an observable tumor phenotype is not unexpected. These data observations suggest that the additional expression of ER α does not appreciably accelerate tumor onset in these strains. On the contrary, ER α /NeuNDL2-5 mice developed multifocal mammary tumors with an average tumor onset of 173 days (Table 2).

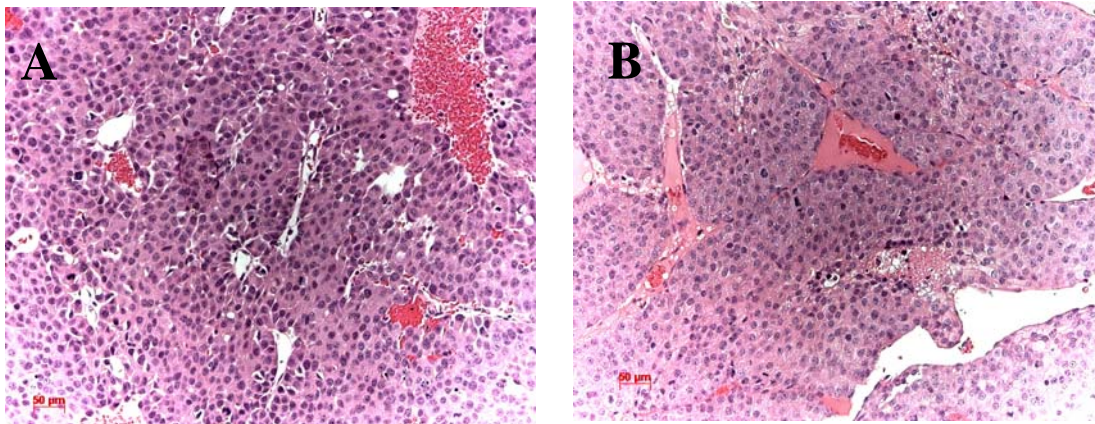
Mammary specific expression of ER α and activated Neu fail to cooperateto accelerate mammary tumor induction.

Table 2 : Tumor onset in bigenic animals compared to activated Neu transgenic mice (NeuNDL2-5)

Genotype	Tumor onset (days)	Total number of animals
aER α /NeuNDL2-5	173 \pm 28	18
NeuNDL2-5	181 \pm 17	11

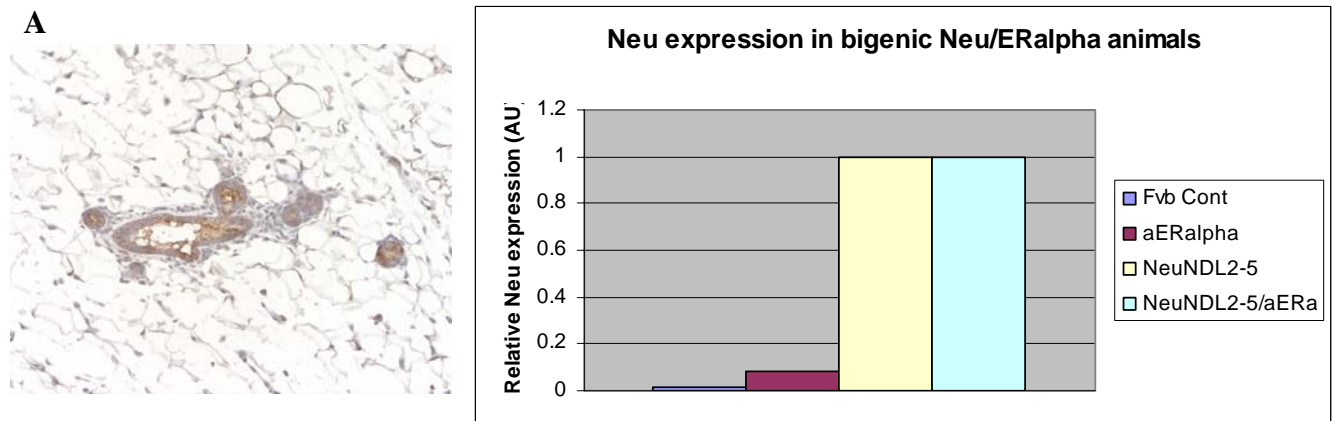
As mentioned above, the bigenic mic co-expressing ER α and Neu developed mammary tumors, however they do so at comparable onset and latency period as the NeuNDL2-5 mice. These observations indicate that ER α does not cooperate with Neu to accelerate tumor induction. Histological analyses through H&E staining studies of paraffin embedded sections of these tumors failed to reveal any difference with tumors from NeuNDL2-5 mice in their pathological phenotypes (Figure 1).

Figure 1 : H&E staining of paraffin embedded sections of (A) NeuNDL2-5 and (B) ER α /NeuNDL2-5 mammary tumors

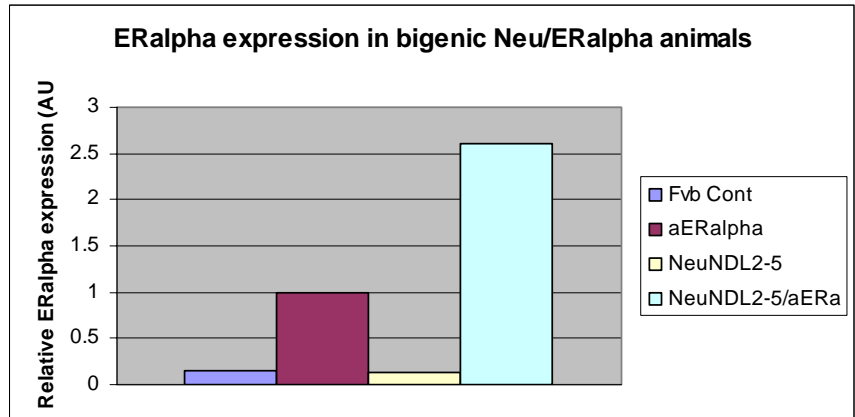
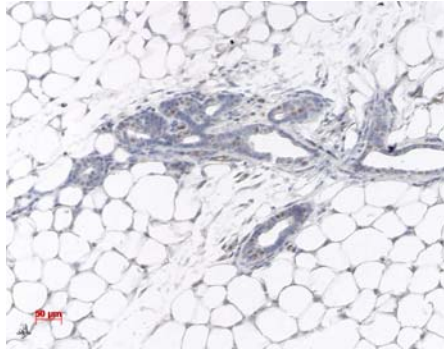


Tumor onset did not significantly differ between NeuNDL2-5 mice (181 days) and ER α /NeuNDL2-5 mice (173 days). The lack of accelerated tumor phenotype is not due to lack of expression of ER α or Neu as the bigenic mammary glands expressed elevated levels of Neu and ER α transcripts compared to FVB control tissues as shown in our previous report and figure 2.

Figure 2 : (A) Immunohistochemical staining of a paraffin embedded section of a virgin mammary gland extracted from an ER α /NeuNDL2-5 mouse using Neu antibodies and relative expression of the Neu transgene using real-time RT-PCR. (B) Immunohistochemical staining of a paraffin embedded section of a virgin mammary gland extracted from an ER α /NeuNDL2-5 mouse using ER α antibodies and relative expression of the ER α transgene using real-time RT-PCR.



B

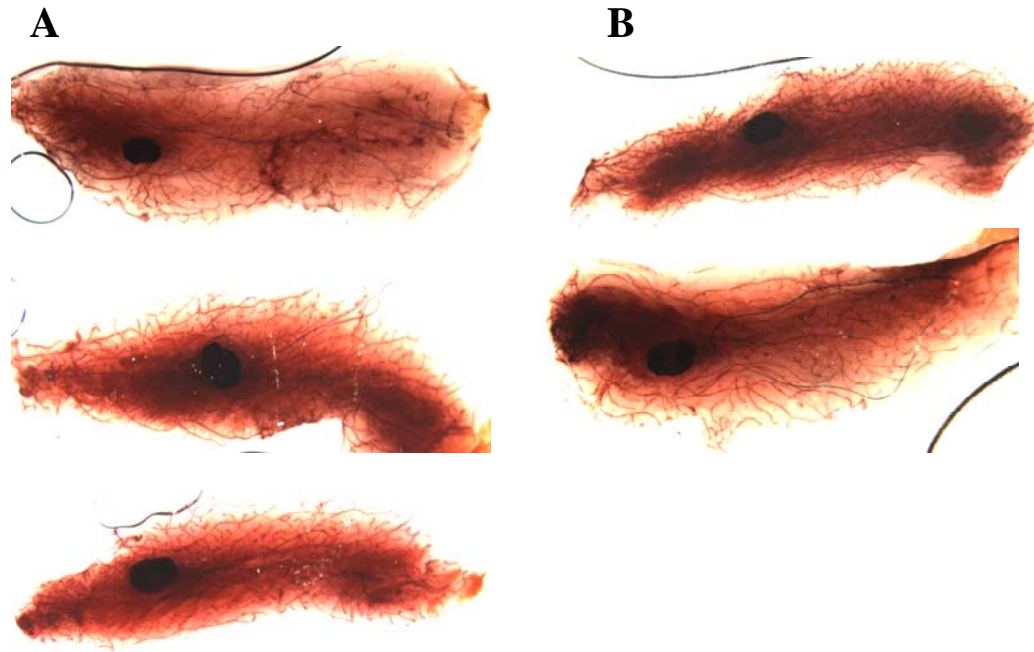


Activated c-Src and ER α do not cooperate to accelerate mammary tumor progression

Although co-expression of activated c-Src and ER α did not result in acceleration of mammary tumor induction, it is conceivable that mammary specific expression of ER α could alter the induction of mammary epithelial hyperplasias typical of MMTV/activated c-Src mice [8]. To further explore whether co-expression of c-Src and ER α might alter mammary gland hyperplastic phenotype observed in the MMTV-src mice, we performed wholemount analyses on 5 months old MMTV/activated c-Src and bigenic MMTV/activated c-Src and ER α strains.

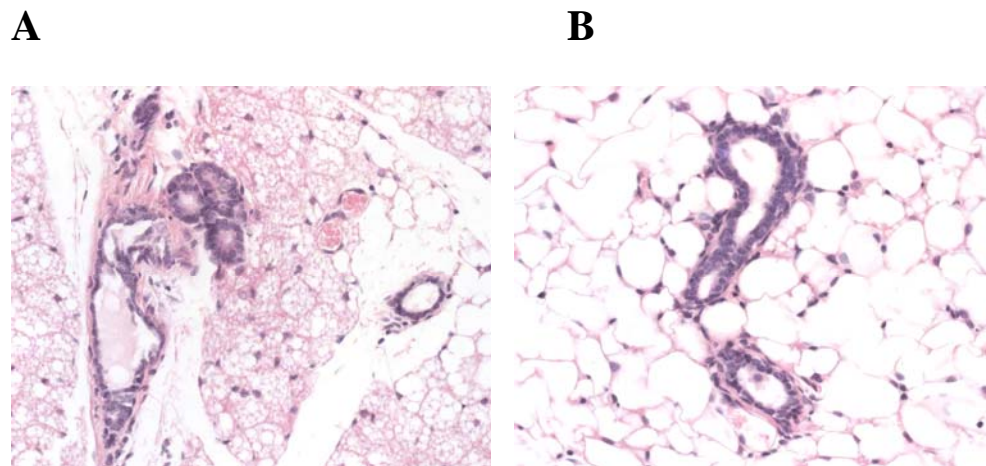
As shown on figure 3, wholemounts taken from Src2 and bigenic aER α /Src 2 mice did not show significant phenotypic differences.

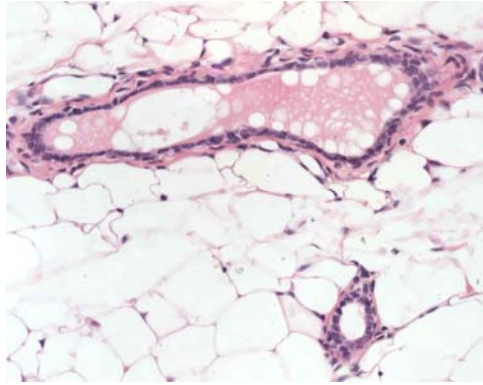
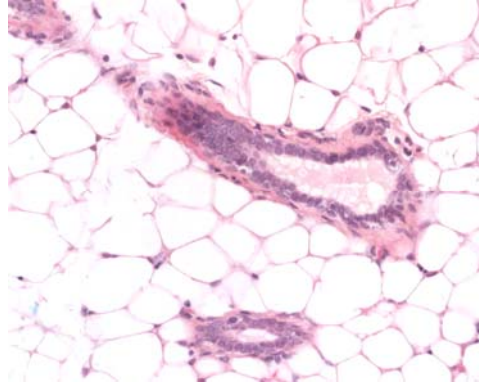
Figure 3 : Wholemounts of mammary glands from virgin aER α /Src 2(A) and Src2 (B) mice at about 5 months of age



These results were confirmed through histological analyses using H&E stained paraffin embedded sections of mammary glands taken from these mice (Figure 4).

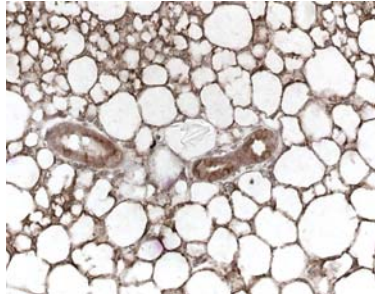
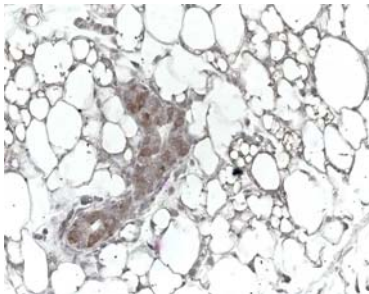
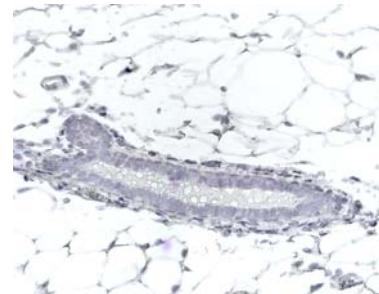
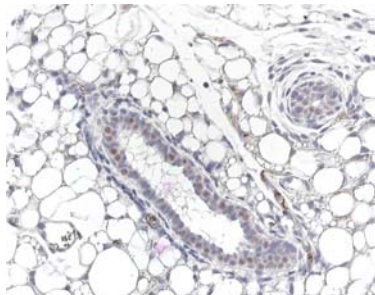
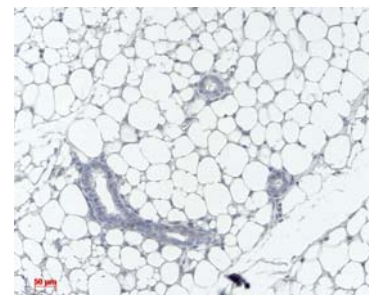
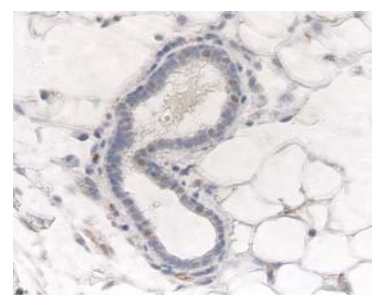
Figure 4 : H&E staining of paraffin embedded sections of mammary glands from (A) aER α , (B) Src 2, (C) aER α /Src 2 and (D) Fvb mice.



C**D**

To confirm that the lack of any observable cooperation between c-Src and ER α was not due to lack of co-expression of ER α and activated c-src, we performed immunohistochemical analyses on mammary histological sections from these strains with ER α and c-Src specific antibodies. The results revealed that both transgenes were expressed in the mammary epithelium of the bigenic animals (Figure 5). Thus the observed lack of cooperation between activated c-src and ER α was not due to lack of expression of the respective transgenes.

Figure 5: Immunohistochemical staining of paraffin embedded sections of mammary glands from virgin mice using specific antibodies directed against Src (A) or ER α (B). The genotype of the mice is noted below each picture.

A**Bigenic aER α /Src 2****Src 2****aER α** **B****Bigenic aER α /Src 2****Src 2****aER α**

It has been shown previously that if overexpression of c-src in Src2 mice is sufficient to induce mammary epithelium hyperplasias, tumors that develop in these strains arise stochastically after a long latency period of about 345 days [8]. Therefore, our results suggest that the concomitant expression of an activated form of ER α in this strain of mice does not accelerate mammary tumor formation unlike we anticipated.

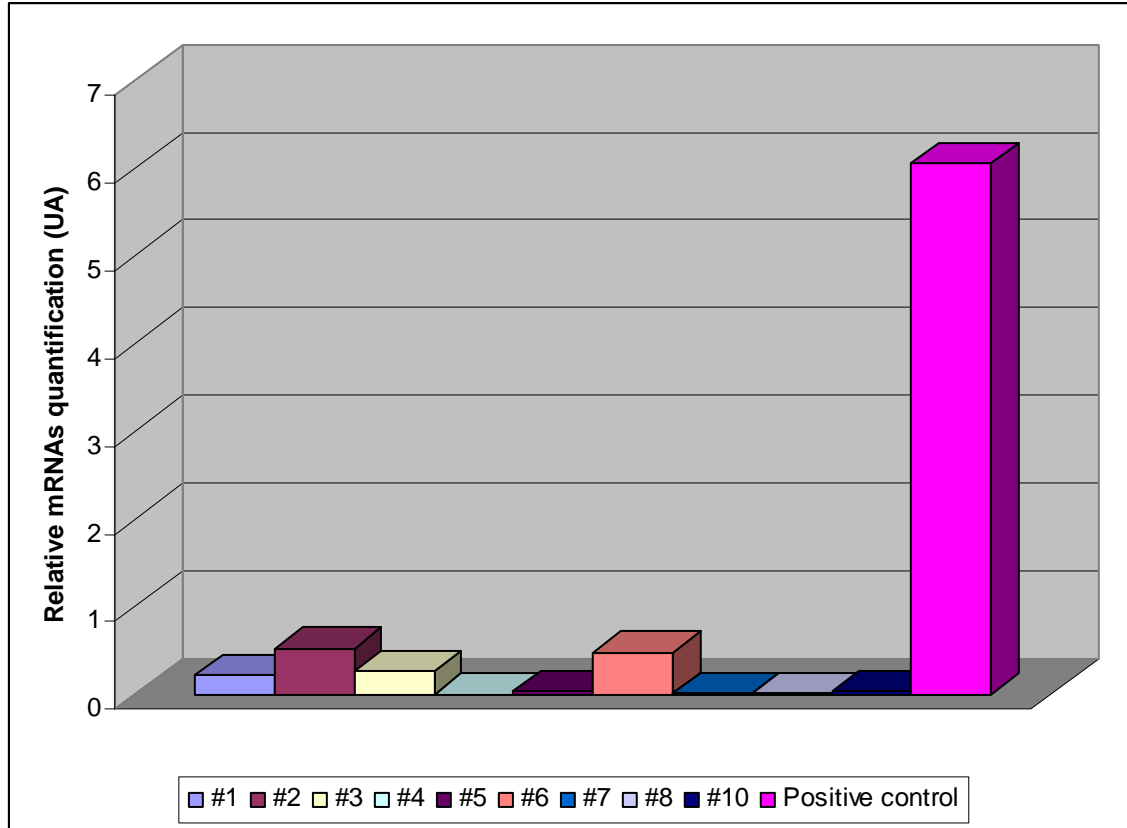
Transgenic mice carrying activated Akt and ER α fail to develop mammary tumors

As mentioned above a number of bigenic ER α /Akt 7 mice have been generated and monitored for tumor formation. These mice did not develop any mammary tumors during the period of time that they were monitored. Since the Akt7 mice (only overexpressing Akt-1) do not develop mammary tumors on their own [6], these data suggest that like for c-Src, and Neu , mammary specific expression of ER α had little impact on the tumor phenotype. Given the lack of observable phenotype, these crosses were not further characterized.

Generation of transgenic mice expressing the ER coactivator amplified in breast cancer, AIB-1

As described in our initial research proposal, we generated transgenic mice harboring the ER coactivator amplified in breast cancer I (AIB-1) by injecting the mouse AIB-I cDNA cloned into an MMTV-based expression and followed by an SV40PolyA signal into one cell mouse embryos. After two rounds of microinjection, we generated 10 mouse founder lines. Exogenous expression of AIB-1 in these 10 different strains has been checked by real-time RT-PCR (Figure 6). None of the ten founder lines did show expression of the exogenous AIB-1 cDNA when compared to a mouse strain positive for SV40polyA expression.

Figure 6 : Relative quantification of exogenous AIB-1 mRNAs through real-time RT-PCR using specific SV40 PolyA primers. Founders animals are numbered #1 to #10; positive control has been prepared from a mouse strain previously characterized for significant expression of SV40polyA.



Given that the Brown laboratory has already published a transgenic mouse expressing AIB-1 in the mammary epithelium that develops high rate of mammary tumor formation [9], we plan to obtain this published strain to determine if ER α can cooperate with AIB-1.

Key Research Accomplishments

- 1- Determination of the average tumor onset of aER α /NeuNDL2-5 mice
- 2- Characterization of bigenic aER α and ErbB-2 tumor phenotype
- 3- Generation of a tri-genic strain co-expressing aER α under the control of the MMTV promoter and activated Neu under the control of its endogenous promoter
- 4- Generation of bigenic mouse strains expressing aER α along with c-Src or Akt-1.
- 5- Generation of 10 mouse founder lines harboring the ER coactivator, AIB-I, cDNA in a MMTV-based expression vector.

Reportable Outcomes

Characterization of MMTV-Neu/ER α mouse models for tumor onset and tumor morphology.

Conclusions

We generated cohorts of transgenic mouse strains expressing an activated form of ER α in the mammary epithelium along with activated Neu, c-Src or Akt-1. The ER α /Src2 and ER α /Akt7 mouse strains did not develop any mammary tumors during the course of the studies. The ER α /NeuNDL2-5 mouse strain develop mammary tumors with an average tumor onset of 173 days that is not significantly different for the average tumor onset of NeuNDL2-5 mice (181 days). Moreover, both types of tumors show an identical pathological phenotype. These results suggest that expression of an activated form of ER α specifically in the mammary epithelium does not influence ErbB-2 induced mammary tumorigenesis nor does it act synergically with c-Src or Akt-1 to accelerate mammary tumor formation in the conditions we tested.

References

1. **Migliaccio, A., et al.**, Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. *Embo J*, 1998. 17(7): p. 2008-18.
2. **Muthuswamy, S.K. and W.J. Muller**, Activation of Src family kinases in Neu-induced mammary tumors correlates with their association with distinct sets of tyrosine phosphorylated proteins in vivo. *Oncogene*, 1995. 11(9): p. 1801-10.
3. **Muthuswamy, S.K. and W.J. Muller**, Direct and specific interaction of c-Src with Neu is involved in signaling by the epidermal growth factor receptor. *Oncogene*, 1995. 11(2): p. 271-9.
4. **Siegel, P.M., et al.**, Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. *Embo J*, 1999. 18(8): p. 2149-64.
5. **Andrechek, E.R., et al.**, Gene expression profiling of neu-induced mammary tumors from transgenic mice reveals genetic and morphological similarities to ErbB2-expressing human breast cancers. *Cancer Res*, 2003. 63(16): p. 4920-6.
6. **Hutchinson, J. et al.**, Activation of Akt (protein kinase B) in mammary epithelium provides a critical cell survival signal required for tumor progression. *Mol. Cell Biol.*, 2001. 21(6): p.2203-12.
7. **Andrechek, E.R. et al.**, Amplification of the Neu/erbB-2 oncogene in a mouse model of mammary tumorigenesis. *Proc. Natl. Acad. Sci USA*, 2000. 97(7): p 3444-9.
8. **Webster, M.A et al.**, Induction of mammary epithelial hyperplasias and mammary tumors in transgenic mice expressing a murine mammary tumor virus/activated c-*src* fusion gene. *Proc. Natl. Acad. Sci USA*, 1995. 92: p. 7849-7853.
9. **Torres-Arzayus, M.I. et al.**, High tumor incidence and activation of PI3K/Akt pathway in transgenic mice define AIB-1 as an oncogene. *Cancer Cell.*, 2004. 6(3): p.263-74.